

Supplementary Appendix

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Supplement to: Mandel-Brehm C, Dubey D, Kryzer TJ, et al. Kelch-like protein 11 antibodies in seminoma-associated paraneoplastic encephalitis. *N Engl J Med* 2019;381:47-54. DOI: 10.1056/NEJMoa1816721

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Kelch-like protein 11 antibodies in seminoma-associated paraneoplastic encephalitis

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SUPPLEMENTARY METHODS

Patient Enrollment and Data Collection

All patient cases were evaluated by board-certified neurologists at Mayo Clinic or the University of California San Francisco (UCSF). Patients 11 and 13 were enrolled in a research study at UCSF (protocol number 13-12236) for pathogen and autoantibody detection for patients with idiopathic neuroinflammation. All other patients (patients 1-10 and 12) were enrolled at the Mayo Clinic and gave consent for the passive use of their medical record for research purposes (protocol numbers 08-007810, 08-007846). Electronic and paper medical records of the thirteen anti-KLHL11 encephalitis cases were reviewed for demographic details, clinical data, laboratory results, and outcome at last follow-up.

Programmable Phage Display

In order to search for antibodies targeted across the human proteome, we adapted the previously developed T7 peptidome phage display library (T7-Pep) that was used together with the phage immunoprecipitation sequencing (PhIP-Seq) methodology to comprehensively detect autoantibodies.^{1,2} Our library consisted of 731,724 overlapping 49 amino acid peptides with a 25 amino acid overlap covering all protein coding regions of the human genome, including all protein isoforms (<https://github.com/derisilab-ucsf/PhIP-PND-2018>). One milliliter of the T7 bacteriophage library (10^{10} plaque forming units) was incubated with CSF or serum, and patient antibodies were captured using protein A/G magnetic beads (Thermo Fisher) and washed. Antibody-bound phage were eluted and amplified in *E. coli* before a second round of immunoprecipitation (IP) followed by DNA extraction and next-generation amplicon single-end

50 nucleotide sequencing on an Illumina MiSeq instrument. Sequencing reads were aligned to the reference human proteome library, and peptide counts were normalized by converting raw read counts to percentage of total reads per sample (Supplementary Table 1). Peptide- and gene-level enrichments were generated by dividing normalized counts by the mean of corresponding normalized counts from a cohort of non-inflammatory control sera (n=28). All samples were run within the same experiment.

Inventory of Antibodies

For all experiments, human CSF or serum was used as primary antibody and visualized using secondary antibodies to goat anti-human IgG-Alexa 568 (Thermo Fisher, A-21090). For all *in vitro* overexpression experiments, rabbit anti-Flag (Cell Signaling Technology, 14793S) or mouse anti-Flag (Sigma, F3165) was used to identify KLHL11. For all *in vivo* analyses, commercial anti-KLHL11 antibody (Sigma, HPA054269) was used. Commercial antibody to KLHL11 was validated through cell-based overexpression assays (Supplemental Figure 3). Primary commercial anti-Flag and anti-KLHL11 antibody binding was visualized with a goat anti-rabbit IgG-Alexa 488 secondary (Abcam, ab150081) unless otherwise noted (Supplemental Figure 4). Co-labeling was qualitatively assessed following confocal microscopy imaging.

293T cell-overexpression based assays

Tissue culture. HEK293T cells (ATCC-CRL3216) are plated at 30% density in complete DMEM media (Sigma) with Glutamine, 10% Fetal Bovine Serum (FBS), 50 ug/mL of Streptomycin and 50 I.U./ml of Penicillin. For immunocytochemistry (ICC) experiments, cells were plated on acid washed glass coverslips in 12-well standard tissue culture plates. For molecular experiments,

including immunoprecipitations and western blotting (WB), the cells were plated on standard 10 cm² tissue culture dishes.

Transfections. Following plating, cells were allowed to seed for 24 hours and subsequently transfected with experimental plasmids using a standard calcium phosphate transfection method. For ICC experiments, 500 ng of a flag tagged human KLHL expressing plasmid (Origene Cat: RC205228) or 500 ng of a GFP expressing plasmid (Origene Cat: PS100010) were transfected per each well of the 12 well plate. For molecular experiments, one microgram of KLHL11 expressing plasmid was transfected per 10 cm² dish. Twenty-four hours following transfection cells were harvested with respect to standard ICC or molecular protocols.

Immunocytochemistry. Twenty-four hours following transfection, cells were washed once in cold 1X PBS and fixed in 4% Paraformaldehyde for 15 minutes at room temperature. Staining protocol: Following washing with 1X PBS, cells were permeabilized and blocked for one hour at room temperature in Blocking buffer (1X PBS, 10% Goat Serum, 0.1% Triton). Following blocking, cells were incubated in one mL of primary antibody buffer (1X PBS, 10% Goat Serum, 0.1% Triton) containing one of the following: Patient IgG (CSF 1:1000, Serum 1:10,000), commercial anti-KLHL11 IgG (1 ug/mL) or commercial anti-FLAG IgG (1 ug/mL). Primary blocking buffer without antibody added was used as a secondary only control. Cells were incubated in primary antibody for 2 hours at room temperature or 4 degrees overnight. Cells were washed 3X in PBST (1X PBS with 0.1% Triton-X) and incubated with secondary antibodies for 1 hour at room temperature, protected by light. To detect human antibodies, anti-Human IgG Alexa-568 was used. To detect rabbit or mouse commercial antibodies, anti-Rabbit IgG or Anti-Mouse IgG Alexa 488 was used. Following secondary antibody incubation, cells

were washed 4X in PBST and mounted on coverglass for microscopy. DAPI was added during mounting using standard DAPI Fluormount-G (Southern Biotech).

Immunoprecipitations and Western Blot. Twenty-four hours following transfection with KLHL-flag plasmid, cells were washed once in cold 1X PBS and one mL of cold RIPA (50 mM Tris-HCL pH 7.5, 150mM NaCL, 1.0% Triton-X, 0.1% SDS, Roche protease inhibitor tablet) was added. Cells were harvested with rubber policemen, lysates were aliquoted into Eppendorf tubes and allowed to lyse in RIPA for an additional 30 minutes at 4 degrees with gentle agitation. Lysates were subsequently spun for 30 minutes at 16,000 g in a tabletop centrifuge at four degrees. Supernatants were then diluted to 1mg/mL protein concentration using Tris-NP40 (50 mM Tris-HCL pH 7.5, 1.0% NP40, 150mM NaCL) and used as lysates for immunoprecipitations.

For each IP, 500 microliters of the 1mg/mL 293T cell lysate was used and one of the following was added per condition: Patient IgG (CSF 1:500, Serum 1:1000), commercial anti-KLHL11 antibody (1 ug/mL). A no antibody condition was used for a protein A/G bead control.

Antibodies were incubated with cell lysate overnight at four degrees with gentle agitation.

Antibodies were captured using protein A/G magnetic beads (Thermo Fisher, Dynabeads) and the beads were washed four times in cold RIPA. IPs were then eluted in 2X laemmli buffer, boiled 95 degrees for 5 minutes, electrophoresed on a 4-12% gradient SDS-PAGE gel (NuPAGE) and transferred to 0.45 micron nitrocellulose for subsequent immunoblotting. To identify the presence of KLHL11-FLAG in the IPs from 293T cell lysates, a rabbit commercial

antibody to anti-FLAG was used for primary antibody and visualized with anti-Rabbit IgG Secondary (LICOR IR800).

Indirect immunofluorescence assay and microscopy, UCSF

Adult, C57B6 male mice were perfused with 4% PFA and the brain was subsequently dissected and cryopreserved in 20% sucrose. Fixed brains were embedded in OCT, slowly frozen on dry ice and stored at -80 until needed. Fixed brains were sectioned on a cryostat at -20 in 12 micron sections onto superfrost plus microscope slides. Sections were dried for 1 hour at RT and then stained with antibodies according to the ICC staining protocol described above. ICC and IHC experiments were visualized using a Nikon Ti Spinning Disc confocal microscope. Image capture settings, including exposure time, laser intensity, aperture, magnification were kept constant for all conditions in the experiment. Image TIFFs were analyzed in Image J and co-localization of RFP and GFP fluorescence was qualitatively determined.

Indirect immunofluorescence assay and microscopy, Mayo Clinic (IFA)

Patient serum and CSF and commercial antibodies were tested on a cryosectioned (4 μ m) composite of adult mouse tissues: cerebellum, midbrain, cerebral cortex, hippocampus, kidney and gut. Sections were fixed using 4% paraformaldehyde for 1 minute, then permeabilized with 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5%, in phosphate buffered saline (PBS, for 1 minute), and then blocked for 1 hour with normal goat serum (10% in PBS). After PBS-rinse, patient specimen was applied (serum was pre-absorbed with bovine liver powder, 1:240 dilution, and CSF was non-absorbed, 1:2 dilution). After 40 minutes, and PBS

wash, a species-specific secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:100) or tetramethylrhodamine (TRITC, 1:100) was applied (Southern Biotechnology Associates, Inc, Birmingham, AL, USA). Cover slips were mounted using ProLong Gold anti-fade medium (containing DAPI; Molecular Probes Thermo Fisher Scientific, USA). Fluorescence images were captured using Olympus BX51 polarizing microscope with Olympus DP73 high-performance Peltier-cooled, 17.28 megapixel camera. Patient specimens yielding positive results were titrated in doubling dilutions to determine the endpoint of autoantibody detection.

For dual staining of mouse tissue, we applied patient serum (1:500) and commercial KLHL11 rabbit polyclonal IgGs (1:800, Catalog # PA5-62944, Thermofischer scientific, USA). Secondary antibodies used were anti-human FITC-conjugated (1:200) and anti-rabbit Alexa Fluor 647-conjugated (1:200, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Confocal images were captured using a microscope (40 × water immersion lens, LSM780; Carl Zeiss AG, Oberkochen, Germany).

Epidemiology

Age- and sex-specific population counts were obtained from the Rochester Epidemiology Project Census of Olmsted County for January 1, 2014 and annually for 1995 through 2015. Prevalence rates were calculated as the number of anti-KLHL11 encephalitis patients by the population count as of January 1, 2014, and were reported per 100,000 people. The incidence rates were calculated as the number of anti-KLHL11 encephalitis patients divided by the total number of person-years at risk and were reported per 100,000 person-years. These rates were adjusted using

the direct method to the sex and age distribution of the total United States population in 2010.³ Both incidence and prevalence rates are reported along with their 95% confidence intervals.

Figure S1. Neuroradiology for Patient 11. The image shown in Panel A was acquired during the patient's initial presentation whereas the image shown in Panel D was acquired 10 months later after further neurologic decline. An axial T2-weighted fluid-attenuated inversion recovery (FLAIR) image of the head (Panel A) revealed a non-enhancing hyperintensity (arrow) around the fourth ventricle, including the right dentate nucleus. An axial T2-weighted FLAIR image (Panel B) showed interval development of left hypertrophic olivary degeneration (arrow).

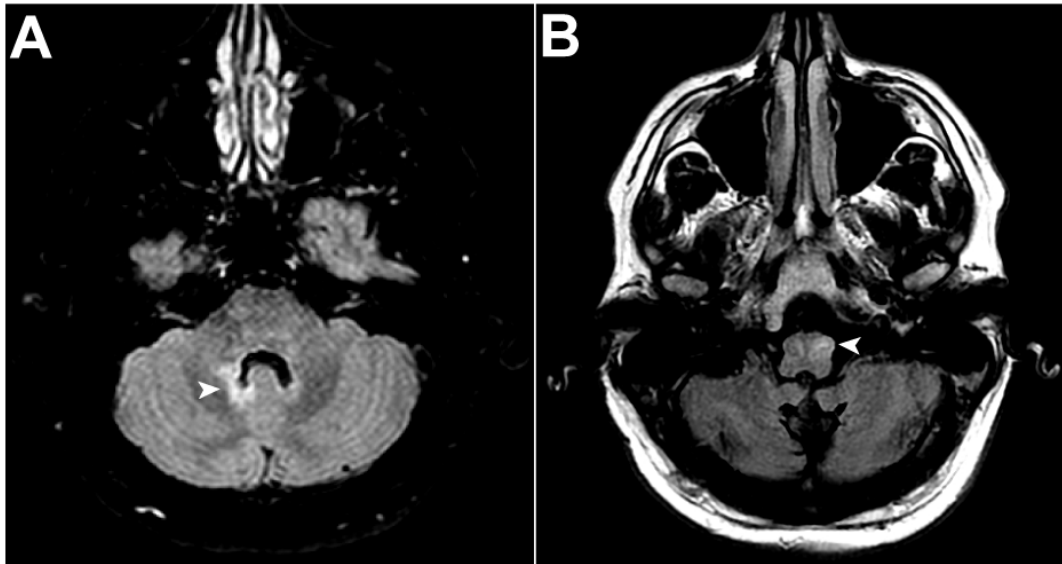


Figure S2. KLHL11-IgG immunofluorescence staining on mouse brain tissues. (Panel A)

Schematic of a sagittal mouse brain section, with graphic summary of IF signal. Patient IgG reactivity is enriched, though not limited to, regions of the brain shaded in red. Shaded areas

designated with a letter have a corresponding representative image in panels B-F. (Panel B)

Periventricular parenchyma. (Panel C) Hippocampus in the vicinity of the dentate gyrus. (Panel

D) Perimeningeal. (Panel E) Vicinity of fourth Ventricle to Dentate Nucleus. (Panel F)

Brainstem. Patient IgG reactivity to mouse brain sections is visualized using anti-Human IgG

Secondary (Alexa 568), DAPI is used to mark nuclei. Magnification 20X. Scale bar denotes 50 microns.

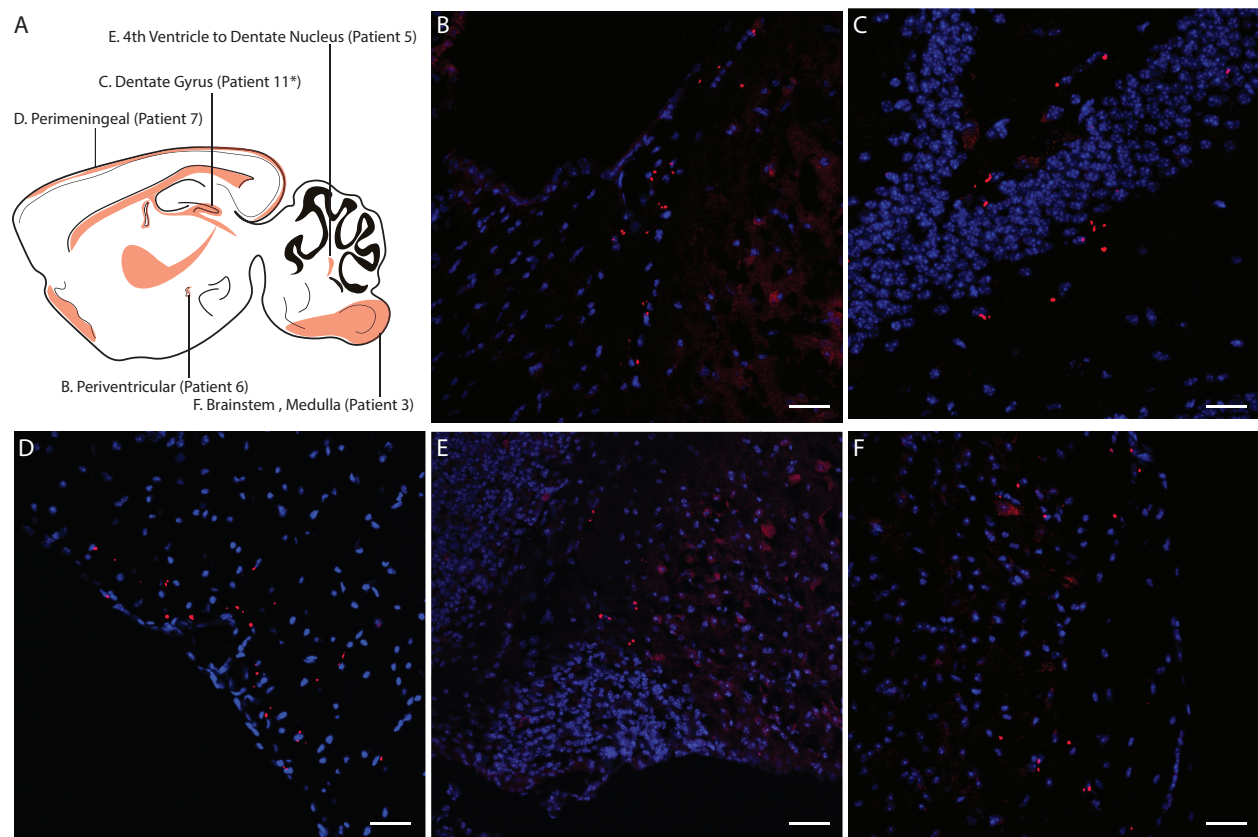


Figure S3. Epitope mapping of KLHL11. Antigenic peptides identified among the KLHL11 patients but not in protein A/G bead negative controls, healthy controls or anti-Ma2 encephalitis controls. Note enrichment of fragment 10 by all KLHL11 patients, including serum and CSF.

*Peptide sequence of fragment 10.

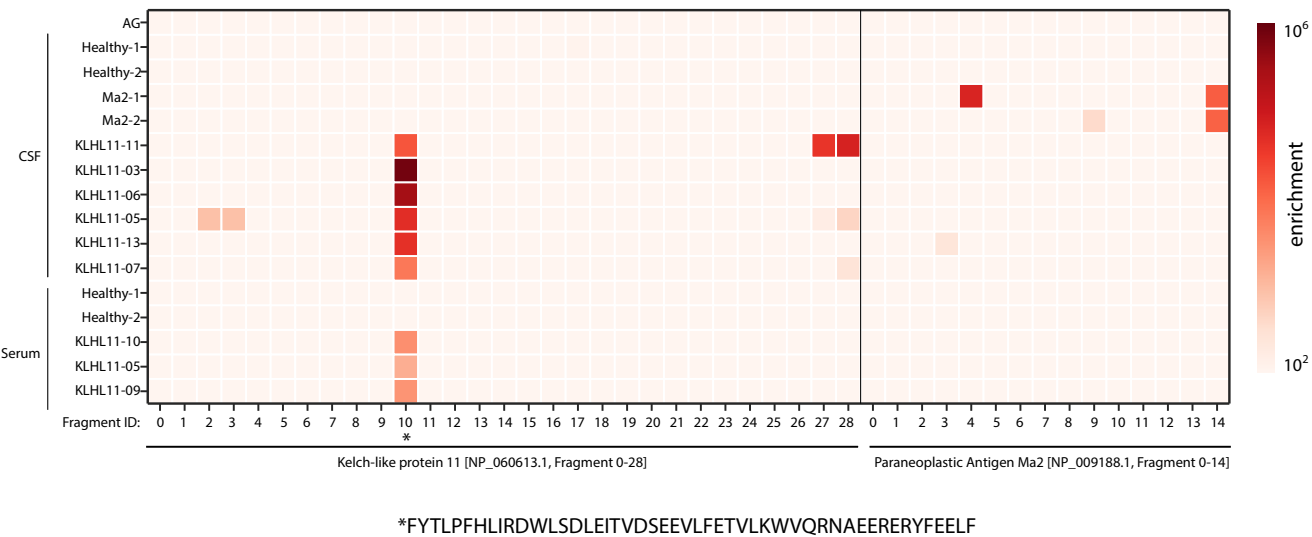


Figure S4. Validation of KLHL11 commercial antibody. Overexpression of human KLHL11-myc-flag expression construct in 293T cells. (Panels A-C) Secondary only control. (Panels D-F) Staining with primary anti-KLHL11 (rabbit) and anti-Flag (mouse) antibodies. Note co-labeling of Flag and KLHL11 signal. To validate the commercial rabbit KLHL11 antibody, to avoid cross reactivity with a rabbit anti-Flag, a mouse anti-Flag antibody was used instead (Sigma, F3165). Primary mouse anti-Flag was visualized with goat anti-mouse IgG-488 (Abcam). Magnification 63X. Scale bar denotes 10 microns.

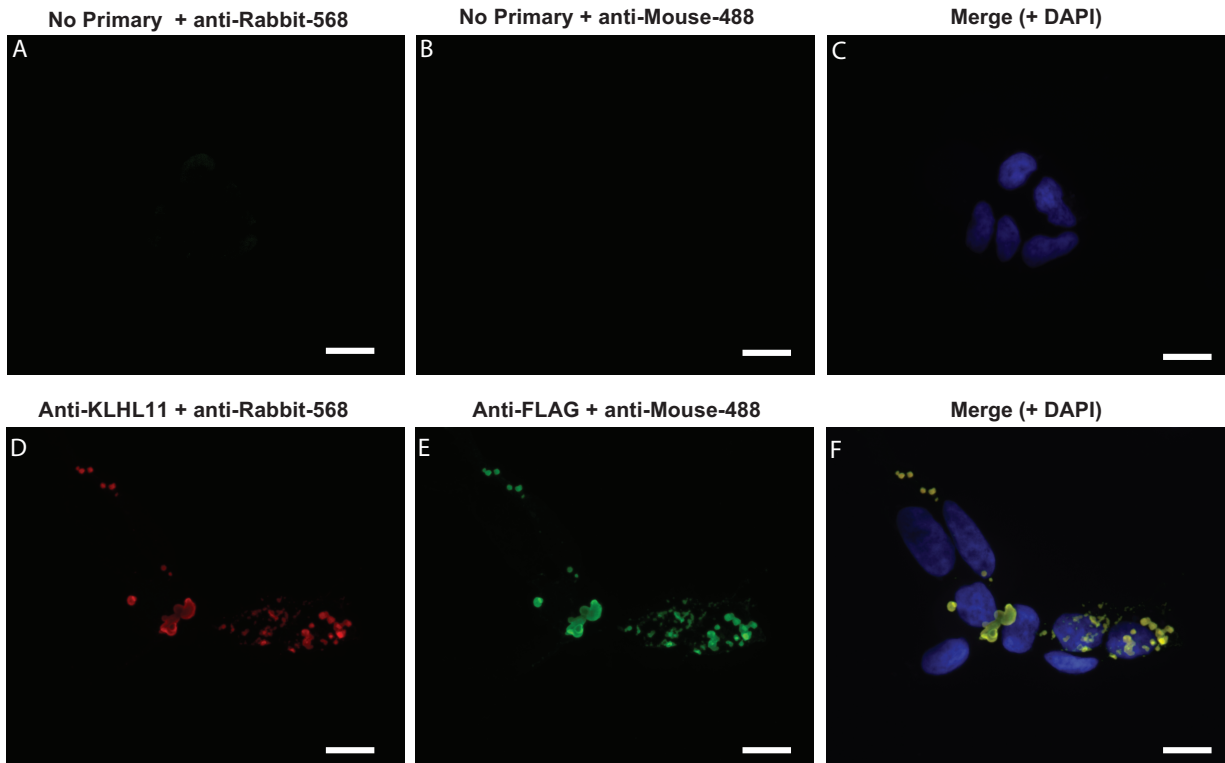


Table S1. Summary of methods by which autoantibodies to KLH11 were identified in each patient.

Patient	Phage Display Immunoprecipitation	Cell-Based Overexpression Assay with Co-labeling	Immunoprecipitation from Cell Lysate	Rodent Brain Immunohistochemistry
1	NT	+ (serum)	NT	+ (serum)
2	NT	+ (serum)	NT	+ (serum)
3	+ (CSF)	+ (CSF)	+ (CSF)	+ (CSF)
4	NT	+ (serum)	NT	+ (serum)
5	+ (CSF, serum)	+ (CSF, serum)	+ (CSF, serum)	+ (CSF)
6	+ (CSF)	+ (CSF, serum)	+ (CSF)	+ (CSF)
7	+ (CSF)	+ (CSF, serum)	+ (CSF)	+ (CSF)
8	NT	+ (serum)	NT	+ (CSF)
9	+ (serum)	+ (serum)	+ (serum)	+ (serum)
10	+ (serum)	+ (serum)	+ (serum)	+ (serum)
11 (Index)	+ (CSF)	+ (CSF, serum)	+ (CSF)	+ (CSF)
12	NT	+ (serum)	NT	+ (serum)
13	+ (CSF)	+ (CSF, serum)	NT	+ (CSF, serum)

Key: NT not tested, CSF cerebrospinal fluid

Table S2. Peptide and gene-level counts for phage display*

[illegible]

*Peptide counts were normalized by converting raw read counts to percentage of total

reads per sample

Table S3: Specificity of KLHL11-IgG immunofluorescence pattern (controls)

Controls tested		(Positive KLHL11 IgG pattern) / (N samples)
Serum samples (533)	Healthy subjects	0/317
	<i>Disease controls</i>	
	Cancer patients without neurological disease	0/69 (including 6 seminomas)
	Ma2 brainstem encephalitis	0/8
	PCA1 IgG (a.k.a anti Yo) associated cerebellar ataxia	0/6
	ANNA2 IgG (a.k.a anti Ri) associated brain-stem encephalitis	0/28
	CLIPPERS	0/17
	Multiple sclerosis	0/38
	Sjögren syndrome	0/15
	CNS systemic lupus erythematosus	0/35
CSF samples (77)	Multiple sclerosis	0/42
	Normal pressure hydrocephalus	0/13
	Acute disseminated encephalomyelitis	0/12
	CLIPPERS	0/5
	CIDP	0/5

Abbreviations: ANNA2 Anti-neuronal nuclear autoantibody type 2, CIDP chronic inflammatory demyelinating polyneuropathy, CLIPPERS chronic lymphocytic inflammation with pontine perivascular enhancement responsive to steroids, CNS central nervous system, CSF cerebrospinal fluid, PCA1 Purkinje cell cytoplasmic antibody type 1.

References

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